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PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY APPLIED TO PEPTIDE SYNTHESIS*

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SUMMARY

A variety of protected synthetic peptides were purified by high-performance liquid chromatography on pre-packed silica gel columns. The compounds varied in protecting groups, amino acid composition and molecular weight. Flow-rates of two to four column volumes per hour were employed, with resultant back pressure of less than 150 p.s.i. A typical column load of 0.2–5 g was purified in 2–5 h.

INTRODUCTION

The rising number of discoveries of a wide spectrum of new peptides which are highly potent and elicit biological responses at the nano- or picomolar level places ever increasing demands on (i) the efficiency and speed of synthesis and (ii) the purity of synthetic peptides. Peptide synthesis by conventional procedures in solution[1] offers the advantage, over solid-phase synthesis, of purification of protected intermediates at various intermittant stages of synthesis[2]. Most procedures such as open column chromatography on Sephadex LH-20[3,4], LH-60[5], or G-50[6] using dimethylformamide or hexamethyl phosphoramide, or silica gel[7] using chloroform or alcohols as eluants, or counter current distribution[8] are relatively slow and require two to four days for completion. The use of organic solvents such as tetrahydrofuran for gel filtration on polystyrene[9] is faster, but large molecular weight differences in components are necessary. High-performance liquid chromatography (HPLC), noted for its speed and efficiency, has thus far mainly been applied to analytical problems in peptide synthesis[10,11].

We have applied HPLC technology to preparative-scale purification of a variety of synthetic peptides using inexpensive pre-packed silica gel columns operated at pressures of 50–150 p.s.i. The compounds varied in amino acid composition, protecting groups and molecular weight. In most cases, the only pre-treatment necessary was dissolution in a suitable solvent and removal of suspended material. A typical chromatogram was completed within 2–5 h.

* Dedicated to Professor Helmut Zahn (Technisch Hochschule, Aachen, G.F.R.) on his 60th birthday.

MATERIALS AND METHODS

Pre-packed silica gel 60 (60-Å pore size) columns (Merck, Darmstadt, G.F.R.) were purchased from E.M. Labs. (Elmsford, N.Y., U.S.A.). Two sizes were used, *viz.* the "A" column, 20 × 1 cm I.D., and the "C" column, 43 × 3.8 cm I.D. The smaller column was used analytically to scan the solvent systems. The preparative separations were carried out on size "C" columns. A flow-rate of 5 ml/min was used with the former, and one of 10 ml/min with the latter, at pressures between 50 and 150 p.s.i. Although column failure by rupture of the glass has not been observed, the columns should be covered with $1\frac{1}{2}$ in. I.D. Tygon tubing, split lengthwise, as a safety measure.

Samples were loaded with a needle-less hypodermic syringe of suitable size, connected to a three-way slide valve via a Luer adapter (Laboratory Data Control) The valve allowed the column to be connected either to the syringe or to the pump. After loading had been completed, the valve was changed to connect the pump to the column and elution commenced.

Reagent-grade solvents were used and mixed on a volume-to-volume basis. Eluant systems were selected on the basis of mixture solubility and prior thin-layer chromatographic (TLC) separations. For those materials soluble in chloroform, elution was begun with chloroform or the even less polar 1-chlorobutane and continued with increasing concentrations of ethanol until the product eluted. Chloroform-insoluble mixtures were loaded in the least polar solvent practical, *e.g.*, chloroform–acetic acid (1:1), and eluted with solvent blends related to the TLC system used. The eluant desired was one where the separation was relatively complete, and the product eluted in less than ten column volumes of solvent. An excellent aid to solvent selection is found in Snyder's paper[12]. The convex gradients employed were generated from a simple two-chamber apparatus having a sealed mixing chamber. The device, fabricated from a reagent bottle and a separatory funnel (details upon request), was attached to the inlet of the pump, and therefore was at only atmospheric pressure.

Various pumps, capable of reaching a flow-rate of 10 ml/min at 100–150 p.s.i. back pressure, were used, *e.g.*, the Milton Roy miniPump, Laboratory Data Control CMP-3, or Beckman Accu-Flo pumps. Connecting tubing was 1/16 in. O.D. PTFE, fitted with 1/4-28 end connectors (available from Laboratory Data Control and others).

Eluted components were detected with an ultraviolet absorbance monitor, either at 254 or 280 nm, using an 0.5-mm path cell (Spectra Physics Model 220). For those materials without sufficient absorbance at these wavelengths, an infrared absorbance monitor, Miran I (Wilks Scientific Corp.) equipped with a 3-mm path length flow cell having CaF_2 windows, was used at a 5.8-μm setting. Small fractions, 1/10 column void volume (20 ml) or less, were collected. This helped to obtain pure material from poorly resolved peaks.

The homogeneity of the product peak, and limits of fraction pooling were determined by TLC screening. A few microliters of selected fractions were spotted on a silica gel plate, the plate was developed in a suitable solvent system, and the spots visualized with chlorine–tolidine reagent[13]. Fractions identified as pure product were then pooled for isolation. Fractions containing product contaminated with a small amount of other materials were sometimes pooled for re-chromatography in the same

solvent system. The purity of isolated products was confirmed by standard criteria, *i.e.*, elemental analyses, amino acid composition, NMR and IR spectroscopy.

After each use, the column was washed, preferentially in reverse direction, with five to ten column volumes of 50% ethanol in chloroform to remove polar materials, then equilibrated with chloroform.

RESULTS AND DISCUSSION

It was the purpose of this study to explore whether the speed and efficiency of HPLC could be utilized for the purification of protected peptides on a preparative scale. There is great need for time-saving measures in conventional peptide synthesis in solution which is exceedingly time-consuming. Furthermore, demands are increasing on the purity of synthetic peptides for biological, clinical, or physicochemical studies. Prospects of obtaining free peptides in analytically pure form are substantially higher if their protected precursors have been rigorously purified.

Any routine use of HPLC in peptide synthesis depended on meeting four requirements:

(a) solvent systems capable of complete separation of protected peptides from starting components, by-products of coupling reactions, and undesired side products within short periods of time

(b) sufficient fractionation power to provide analytically pure products

(c) ability to use powerful solvents of high polarity, especially for partially protected and larger peptides of increasingly poor solubility

(d) sufficient column capacity for gram-scale chromatography and column reuse ability.

The examples described below show that suitable conditions have been developed which meet these requirements for up to octapeptides by using individual eluant systems for each compound. A preparation of the tripeptide Z-Phe-Thr(Bu^t)-Ser(Bu^t)-OMe* was shown by TLC (chloroform–methanol–acetic acid, 40:1:0.2) to contain at least six contaminants, *e.g.*, the starting components Z-Phe-OTcp and H-Thr(Bu^t)-Ser(Bu^t)-OMe, the by-product 2,4,5-trichlorophenol and unidentified side-products. Five grams of the mixture, dissolved in 1-chlorobutane, were loaded onto a "C" column (43 × 3.8 cm). Elution was carried out sequentially with 1-chlorobutane, chloroform, 1% ethanol in chloroform, and 5% ethanol in chloroform (Fig. 1). The complex mixture was well-separated, and the product (2.9 g, fractions 72–118) was recovered within 4 h. Solvent evaporation produced a colorless amorphous powder which was homogeneous by TLC and microanalytical data, in close agreement with theoretical values.

A preparation of the pentapeptide Z-Lys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-OMe, obtained by condensing Z-Lys(Boc)-ONp with the tetrapeptide methyl ester, was chromatographed in a similar system (Fig. 2), but loading and elution were begun with chloroform. From 4.5 g of mixture, loaded in 27 ml of chloroform, 4.3 g of analytically pure product were recovered after 2½ h.

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* Abbreviations (*J. Biol. Chem.*, 247 (1972) 977): Acm = acetamidomethyl; Boc = *tert.*-butyloxycarbonyl; Bu^t = *tert.*-butyl; OBu^t = *tert.*-butyl ester; OMe = methyl ester; ONp = *p*-nitrophenyl ester; OTcp = 2,4,5-trichlorophenyl ester.

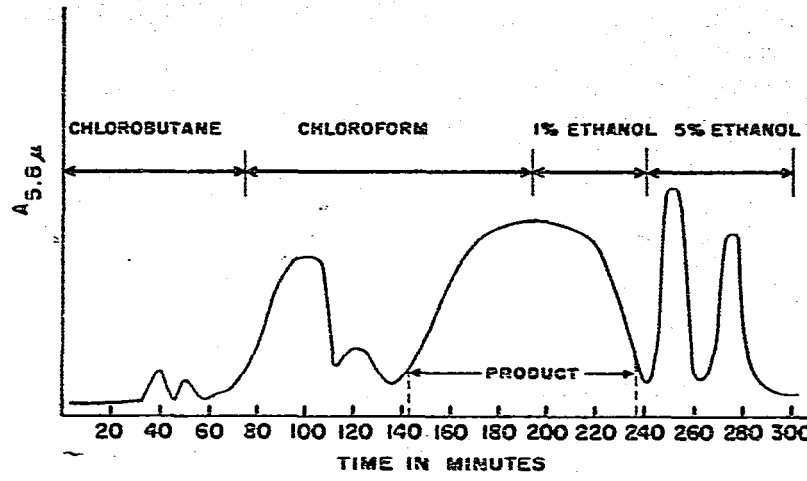

Fig. 1. Chromatography of Z-Phe-Thr(Bu^t)-Ser(Bu^t)-OMe on silica gel 60. The sample (5 g) was loaded in 20 ml of 1-chlorobutane onto a 43 × 3.8 cm I.D. column equilibrated with that solvent. Elution was carried out at 10 ml/min with the solvent program shown. The eluate was monitored at 5.8 μm. Fractions were collected at 2-min intervals.

Condensation of the hexapeptide azide[14], obtained from Z-Trp-Lys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-$NHNH_2$, with S-acetamidomethyl-L-cysteine yielded a product mixture (185 mg) of limited solubility. It was dissolved in glacial acetic acid (1 ml), diluted with chloroform (1 ml), and loaded onto an "A" column (20 × 1 cm), equilibrated with chloroform–2-propanol–acetic acid (91:7:2). Isocratic elution with the same system (Fig. 3), provided the desired heptapeptide derivative in less than

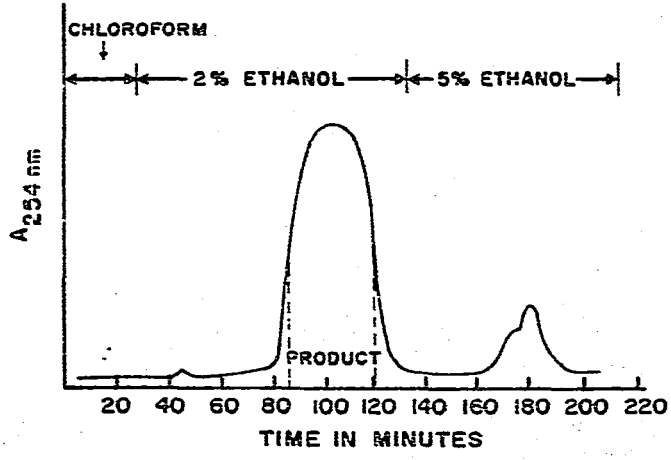

Fig. 2. Chromatography of Z-Lys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-OMe on silica gel 60. The sample (4.5 g) was loaded in 27 ml of chloroform onto a 43 × 3.8 cm I.D. column equilibrated with that solvent. Elution was carried out at 10 ml/min with the solvent sequence indicated. The eluate was monitored at 254 nm. Fractions were collected at 2-min intervals.

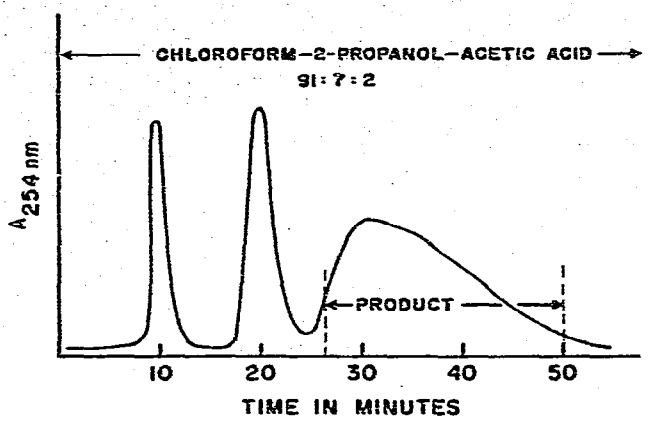

Fig. 3. Chromatography of Z-Trp-Lys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys(Acm)-OH on silica gel 60. The 185-mg sample was dissolved in 2 ml of acetic acid–chloroform (1:1) and loaded onto a 20 × 1 cm I.D. column previously equilibrated with chloroform–2-propanol–acetic acid (91:7:2), and eluted with the same system at 1 ml/min. Fractions were collected at 1-min intervals.

2 h. Evaporation yielded an analytically pure product which crystallized (115 mg) from isopropanol–hexane.

Removal of the N$^{\alpha}$-benzyloxycarbonyl group of the heptapeptide by catalytic hydrogenation in liquid ammonia[15,16] yielded a partially protected peptide derivative which was purified to homogeneity by chromatography (not shown) on an "A" column with chloroform–methanol–acetic acid (75:20:51) as an eluant.

The partially protected pentapeptide H-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys-(Bu^t)-OBu^t, with a free α-amino group, was chromatographed using gradient elution.

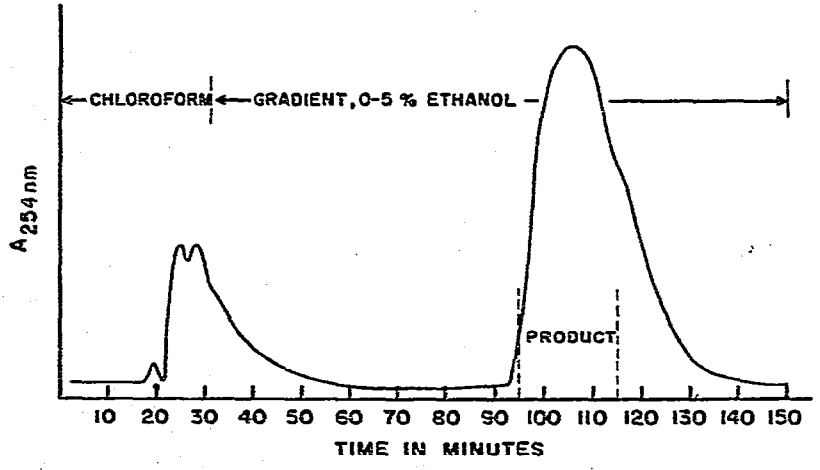

Fig. 4 Chromatography of H-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys(Bu^t)-OBu^t on silica gel 60 using gradient elution The crude sample (2.9 g) was loaded in 10 ml of chloroform onto a chloroform-equilibrated 43 × 3.8 cm I.D. column. Elution was carried out at 8 ml/min with 250 ml of chloroform, followed by a convex gradient to 5% ethanol in chloroform over 2 h. Fractions were collected every 2 min.

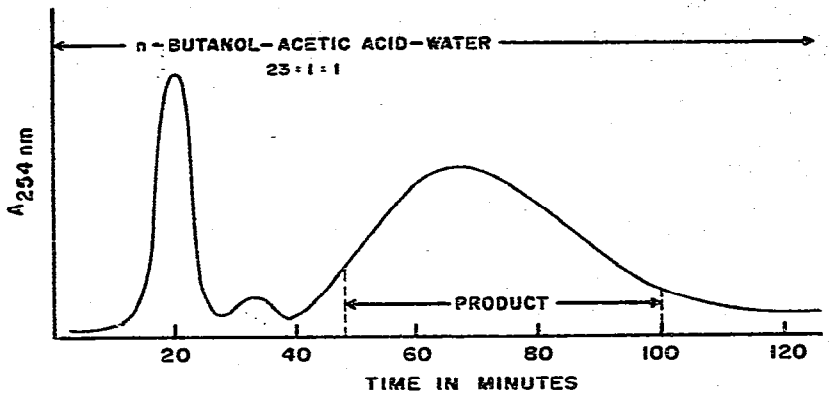

Fig. 5. Chromatography of Z-Pro-Pro-His-Leu-Leu-Val-Tyr-Ser-NHNH-Boc on silica gel 60. The sample, 1.4 g, was loaded as a 10% solution in glacial acetic acid. The 43 × 3.8 cm I.D. column had been equilibrated with the eluant *n*-butanol–acetic acid–water (23:1:1). Elution was conducted at 8 ml/min. Fractions were collected at 1.5-min intervals.

A chloroform solution of the peptide preparation (3.9 g) was loaded onto a "C" column equilibrated with chloroform. Elution was started with 250 ml of chloroform followed by a convex gradient generated from 500 ml each of chloroform and 10% ethanol in chloroform (Fig. 4). Resolution was poor but 2.1 g of homogeneous product were isolated within 2 h from fractions 48–57. The trailing part of the peak (fractions 58–65) containing contaminated product was readily re-chromatographed on the same column using the same solvent system.

The octapeptide, Z-Pro-Pro-His-Leu-Leu-Val-Tyr-Ser-NHNH-Boc, was of low solubility and contaminated with a less polar compound of higher molecular weight, which could not be removed by gel chromatography on Sephadex LH-20[4]. The mixture (1.4 g) was dissolved in 15 ml of glacial acetic acid and loaded onto an "C" column, equilibrated with *n*-butanol–acetic acid–water (23:1:1). Isocratic elution (Fig. 5) with the same solvent system yielded an analytically pure product (0.9 g) within less than 2 h.

These examples have been selected to indicate that satisfactory resolution of complex reaction mixtures can be achieved on a preparative scale within short periods of time. Mixtures have been resolved which resisted fractionation by conventional open-column methods and protected peptides of improved purity have been obtained. Powerful solvents such as glacial acetic acid or dimethylformamide[17] can be used for loading of less soluble products. It is possible to apply crude reaction mixtures after peptide coupling without prior conventional washing*. At the high flow-rates used, product elution has been completed within 2–5 h. Samples of 0.1 g up to 5 g have been purified in single runs. For easy separations (product widely resolved from contaminants) the upper load limit of a "C" column was 10 g.

The efficiency of the column, as measured by the number of theoretical plates[18],

* This has saved additional time and allowed in some cases to complete synthesis and purification within an 8-h work day, provided that fast coupling methods (mixed anhydride or carbodiimide procedures) were used.

is sufficient for most separations. The plate count varied from column to column, but was generally between 200 and 400.

The lifetime of these columns was limited by the amount of insoluble material deposited in the inlet frit. The resultant clogging caused pressure build-up and eventual failure of the column by ejection of the inlet tubing. If samples were carefully filtered through a "fine" porosity glass frit before loading, a dozen or more chromatograms could be run on a single column.

We believe that the procedure described herein for rapid and convenient preparative-scale purification of protected peptides by HPLC on pre-packed silica gel 60 columns could be extended to many other peptides and become a routine procedure in conventional peptide synthesis. Apart from providing products of improved purity, very substantial amounts of time and effort can be saved.

REFERENCES

1 E. Schröder and K. Lübke, *The Peptides*, Vol. 1, Academic Press, New York, 1965.
2 J. Meienhofer, in C. H. Li (Editor), *Hormonal Proteins and Peptides*, Vol. 2, Academic Press, New York, 1973, pp. 48–267.
3 E. Nyström and J. Sjövall, *J. Chromatogr.*, 24 (1966) 208.
4 J. Meienhofer, R. Cotton and E. Atherton, *J. Org. Chem.*, 36 (1971) 3746.
5 A. R. Zeiger and C. B. Anfinsen, *J. Amer. Chem. Soc.*, 95 (1973) 880.
6 I. J. Galpin, G. W. Kenner, S. R. Ohlsen and R. Ramage, *J. Chromatogr.*, 106 (1975) 125.
7 E. Jaeger and M. Deffner, in E. Wünsch (Editor), *Houben-Weyl, Methoden der organischen Chemie, Vol. 15/2, Synthese von Peptiden*, Thieme, Stuttgart, 1974, pp. 492–494.
8 E. Jaeger and M. Deffner, in E. Wünsch (Editor), *Houben-Weyl, Methoden der organischen Chemie, Vol. 15/2, Synthese von Peptiden*, Thieme, Stuttgart, 1974, pp. 572–608.
9 A. B. Russell and M. Szelke, in H. Nesvadba (Editor), *Peptides 1971*, North-Holland, Amsterdam, 1973, pp. 223–225.
10 R. S. Ward and A. Pelter, *J. Chromatogr. Sci.*, 12 (1974) 570.
11 E. Gil-Av, in Y. Wolman (Editor), *Peptides 1974*, Wiley, New York, 1975, pp. 247–256.
12 L. R. Snyder, *J. Chromatogr.*, 92 (1974) 223.
13 H. Zahn and E. Rexroth, *Z. Anal. Chem.*, 148 (1955) 181.
14 J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, 26 (1961) 2333.
15 J. Meienhofer and K. Kuromizu, *Tetrahedron Lett.*, (1974) 3259.
16 K. Kuromizu and J. Meienhofer, *J. Amer. Chem. Soc.*, 96 (1974) 4978.
17 T. F. Gabriel, M. H. Jimenez, A. M. Felix, J. Michalewsky and J. Meienhofer, *Int. J. Peptide Protein Res.*, in press.
18 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968.